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Vasoactive intestinal peptide induces IL-8 production in human colonic epithelial cells via MAP kinase-dependent and PKA-independent pathways

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Abstract

Vasoactive intestinal peptide (VIP) has been shown to be a key regulator of intestinal epithelial functions such as mucus and chloride secretion, paracellular permeability, and cell proliferation. However, its regulatory role in intestinal epithelial chemokine production remains unknown. The aim of this study was (1) to determine whether VIP can modulate intestinal epithelial interleukin-8 (IL-8) production and (2) to identify intracellular mediators responsible for this effect. In the human colonic epithelial cell line HT29-Cl.16E, VIP stimulates IL-8 secretion dose-dependently and IL-8 mRNA level at 10^{-9} M. The protein kinase A (PKA) inhibitor PKI did not abolish the effect of VIP. However, inhibition of the ERK1/2 and p38 MAPK pathways reduced the VIP-stimulated IL-8 secretion and mRNA level. Together, our results showed that VIP stimulates IL-8 production in intestinal epithelial cells via PKA-independent and MAPK-dependent pathways. These data suggest that VIPergic pathways can play an immunomodulatory role in intestinal epithelial cells, by regulating epithelial IL-8 secretion.

Keywords: Vasoactive intestinal peptide; Neuropeptide; Chemokine; IL-8; MAP kinase; Human colonic epithelial cells

Enteric neuromediators are known to regulate mucosal functions, e.g., mucosal blood flow and mucus and electrolyte secretions [1–5]. Among the key neuromediators regulating mucosal functions is vasoactive intestinal peptide (VIP), which induces vasodilatation in the colon, modulates chloride and mucin secretions [2–6], and also controls paracellular permeability and epithelial cell proliferation [7,8]. In addition, recent data show that enteric neuromediators can regulate the secretion of cytokines and chemokines in intestinal epithelial cells. In particular, substance P has been shown to increase intestinal epithelial IL-8 secretion while somatostatin decreases it [9,10]. IL-8 belongs to the CXC chemokine family and is involved in the recruitment of neutrophils, as well as in repair processes in intestinal epithelial cells [11–13].

Regulation of IL-8 epithelial secretion by neuropeptides or cytokines has been shown to involve various intracellular pathways. In particular, substance P-induced IL-8 secretion involves the Rho family small GTPases [9]. Another important signalling pathway in IL-8 production by intestinal epithelial cells upon stimulation with inflammatory mediators such as TNFα or IL-1β involves the mitogen-activated protein kinases (MAPKs) p38 and ERK1/2 [14,15]. In addition, a protein kinase A (PKA)-dependent pathway controls the increase in IL-8-induced gene expression by the prostaglandin PGE2 [16].

Nevertheless, it is currently unknown whether VIP is able to modulate IL-8 production in intestinal epithelial cells. Therefore, the aim of this study was to (1) determine whether VIP can modulate intestinal epithelial IL-8 production at the protein and mRNA levels in the human colonic epithelial cell line HT29-Cl.16E [17], a validated model for assessing the control of epithelial functions by enteric neuromediators [2,5,6,18]; and (2)

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identify intracellular transduction pathways involved in the effect of VIP.

Materials and methods

Cell culture. The HT29-Cl.16E epithelial cell line was grown on porous filters (12-well Transwell Clear, 0.40 μm porosity, Costar, France) and formed monolayers of polarized cells at post-confluence, which differentiated into mucus-secreting cells [17]. Cells were seeded at 1.2×10^6 cells/well in DMEM (4.5 g/L glucose)/10% heat-inactivated FCS (InVitrogen, Cergy-Pontoise, France) and cultured until post-confluence and full differentiation. Experiments were conducted at days 13–16 after seeding. Each experiment was performed at least three times in triplicate. Drugs were dissolved either in deionized water or DMSO. VIP (Sigma, Saint Quentin Fallavier, France) was applied at the indicated concentration to the basolateral compartment for 24 h, except for kinetics studies. In experiments using inhibitors, the PKA peptide inhibitor PKI (5 μg/ml, Sigma), the ERK1/2 inhibitor PD98059 (50 μM, Calbiochem, VWR, Strasbourg, France) or the p38 inhibitor SB203580 (10 μM, Calbiochem) was preincubated to both compartments 1 h before addition of VIP. Controls were performed with the appropriate vehicle.

Measurement of IL-8 protein. After treatment, apical and basolateral culture media were collected separately and IL-8 protein levels were determined using a sandwich-ELISA (BD-Pharmingen, Le Pont de Claix, France) following the manufacturer's instructions. Each sample was assessed in duplicate.

Real time RT-PCR analysis of IL-8 mRNA level. Total RNA extraction from HT29-Cl.16E cells was performed with Tri Reagent (Euromedex, Souffel Weyersheim, France) according to the manufacturer's instructions. For reverse transcription, RNA (5 μg) was combined with 0.5 μg of random hexamers (Promega, Charbonnières, France), transcription buffer (50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, and 10 mM DTT), dNTPs (1 mM each, Promega), RNasin (50 U; Promega), and RNaseH$^-$ M-MLV reverse transcriptase (200 U; Promega) in a total volume of 25 μl. Incubation was performed at 42 °C for 60 min. The amplification conditions of the IL-8, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-actin templates were optimized for the RotorGene 2000 instrument (Ozyme, Saint Quentin en Yvelines, France). PCR amplifications were performed using Titanium *Taq* DNA polymerase (Clontech, Ozyme). The reaction mixture contained 2 μl of the supplied 10× Titanium *Taq* PCR buffer (containing magnesium chloride), 1 μl of a 1/1000 dilution of SYBR Green I (Roche, Meylan, France), 1 μl of each primer (0.4 μM each), 0.4 μl Titanium *Taq* DNA polymerase, 0.5 μl dNTPs (10 mM each; Clontech), and PCR-grade water to a volume of 18 μl. Microtubes (0.2 ml) were loaded with 18 μl of this master mix and 2 μl of the template (cDNA diluted 1/100) and the run was initiated. The cycling conditions were as follows: denaturation for 5 min at 95 °C; amplification for 35 cycles (GAPDH and β-actin) or 40 cycles (IL-8), with denaturation for 5 s at 95 °C, annealing for 15 s at 62 °C for GAPDH and 63 °C for IL-8 and β-actin, and extension for 20 s at 72 °C and for 15 s at the specific melting temperature. To exclude primer–dimer artifacts, fluorescence was measured at a temperature (89 °C for GAPDH, 87 °C for β-actin, and 83 °C for IL-8) above the melting point of primer–dimers and 3 °C below the melting point of the specific PCR products. At the end of each cycle, the fluorescence emitted by the SYBR Green I dye was measured. After completion of the cycling process, samples were subjected to a temperature ramp (from 65 to 99 °C) with continuous fluorescence monitoring for melting curve analysis.

Primers were chosen on separate exons to amplify cDNA but not genomic DNA. The following primers were used:

GAPDH forward 5′-TGAACGGGAAGCTCACTGG-3′
GAPDH reverse 5′-TCCACCACCCTGTTGCTGTA-3′
β-actin forward 5′-CCTTCCTGGGCATGGAGTCCTG-3′
β-actin reverse 5′-GGAGCAATGATCTTGATCTTC-3′
IL-8 forward 5′-CTCTTGGCAGCCTTCCTGATT-3′
IL-8 reversed 3′-TATGCACTGACATCTAAGTTCTT-3′

For each PCR product, a single narrow peak was obtained by melting curve analysis at the specific melting curve temperature, indicating specific amplifications. An external standard curve was generated with serial fivefold dilutions (1/20, 1/100, and 1/500) of the control sample (cDNA from HT29-Cl.16E). The reference curve was constructed by plotting the relative amounts of these dilutions vs. the corresponding C_t (threshold cycle) values. The correlation coefficient of these curves was always greater than 0.99. The amount of IL-8, GAPDH or β-actin transcripts was calculated from these standard curves using the RotorGene software (Ozyme). Samples were tested in triplicate and the average values were used for quantification. For each sample, the ratio between the relative amounts of IL-8 and GAPDH or β-actin was calculated to compensate for variations in quantity or quality of starting mRNA as well as for differences in reverse transcriptase efficiency. GAPDH was used for time-course experiments whereas β-actin was used for the study of MAPK involvement since we observed that the MAPK inhibitors PD98059 and SB203580 decreased GAPDH mRNA levels.

Statistical analysis. Each set of experiments was repeated at least three times. Values are given as means ± SE. Conditions were compared using Student's paired *t* test. One way analysis of variance on the ranks for repeated measures was performed for multiple comparisons. Differences were considered as significant for $p < 0.05$.

Results

VIP dose-dependently increases basolateral IL-8 secretion in HT29-Cl.16E cells

VIP was added to the basolateral side at different concentrations (10^{-11}–10^{-9} M). As shown in Fig. 1, VIP elicited at 24 h a significant, dose-dependent increase in basolateral IL-8 secretion reaching threefold the control level at 10^{-9} M. By contrast, VIP did not modify apical

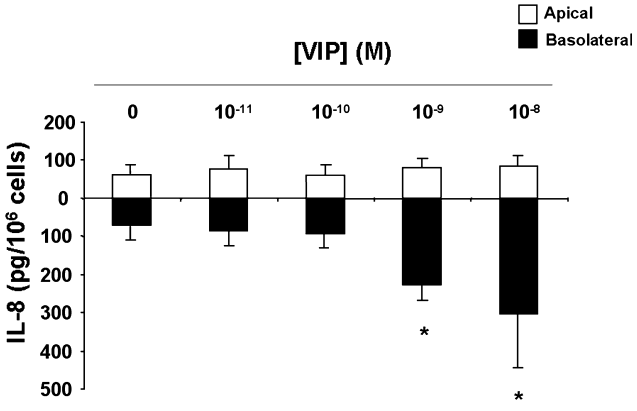

Fig. 1. VIP dose-dependently increased basolateral IL-8 secretion in HT29-Cl.16E. IL-8 measurement by ELISA in apical or basolateral media showed that VIP induced a significant increase in IL-8 basolateral secretion compared to control monolayers at 10^{-9} and 10^{-8} M VIP (24 h incubation). In contrast, VIP did not modify apical IL-8 secretion. Means ± SEM of three different experiments performed in triplicate. *$p < 0.05$.

IL-8 secretion. Furthermore, kinetic studies showed that VIP-induced IL-8 secretion was detected as early as 8 h after VIP addition and was maximal at 24 h (data not shown).

VIP increases IL-8 mRNA levels in HT29-Cl.16E cells

We then determined whether VIP modulated IL-8 mRNA levels, assessed by real time quantitative RT-PCR analysis. As shown in Fig. 2, VIP (10^{-9} M) induced a significant increase in IL-8 mRNA level as compared to control cells. This increase (2.5-fold control level) was significant 8 h after the addition of VIP and was maintained up to 24 h.

IL-8 secretion in HT29-Cl.16E cells is PKA-independent

We then tested whether a PKA-dependent pathway was involved in VIP-induced IL-8 expression. Preincubation of HT29-Cl.16E cells for 1 h with the PKA inhibitor PKI (5 μg/ml) prior to addition of 10^{-9} M VIP did not modify either the VIP-stimulated IL-8 secretion or the basal level of IL-8 secretion (Fig. 3). Similar results were obtained with another PKA inhibitor, H89 (data not shown).

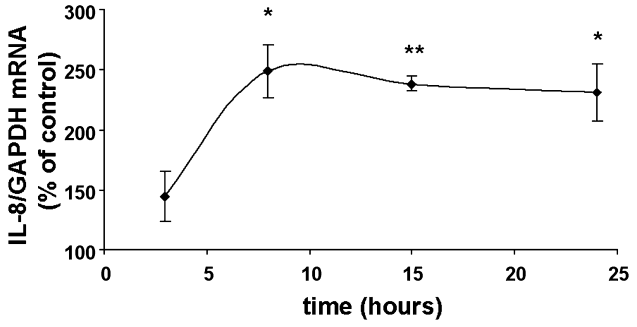

Fig. 2. VIP increased IL-8 mRNA levels in HT29-Cl.16E. Real time quantitative RT-PCR analysis of IL-8 mRNA level normalized to GAPDH showed a significant increase in mRNA level as early as 8 h and until 24 h after addition of VIP (10^{-9} M). Results are normalized to control level for each time point. Means ± SEM of three independent experiments. $*p < 0.05$ and $**p = 0.004$.

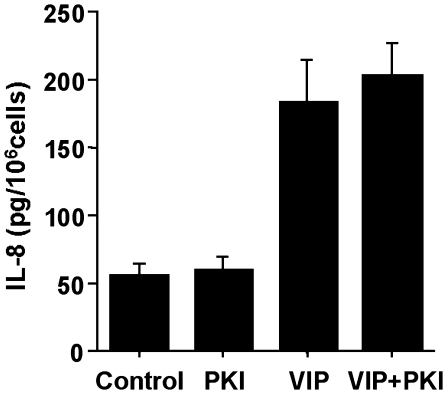

Fig. 3. The VIP-stimulated basolateral IL-8 secretion was PKA-independent. A pre-incubation with the PKA inhibitor PKI (5 μg/ml) did not modify the IL-8 basolateral secretion stimulated by 10^{-9} M VIP. Means ± SEM of three independent experiments performed in triplicate.

ERK1/2 and p38 are involved in VIP-induced IL-8 production

In order to characterize the involvement of MAPK in the VIP-induced IL-8 production, the monolayers were preincubated for 1 h with either the specific ERK1/2 inhibitor PD98059 (50 μM) or the specific p38 inhibitor SB203580 (10 μM) or with both inhibitors, prior to the addition of 10^{-9} M VIP. Fig. 4 shows that PD98059 and

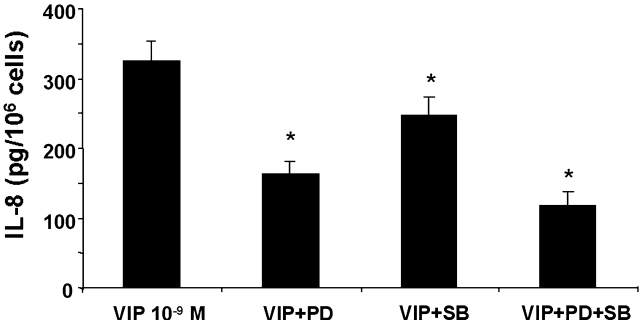

Fig. 4. ERK1/2 and p38 pathways were involved in the VIP-stimulated basolateral IL-8 secretion. The ERK1/2 inhibitor PD98059 (50 μM), the p38 inhibitor SB203580 (10 μM), or both inhibitors significantly reduced the IL-8 basolateral secretion stimulated by 10^{-9} M VIP after 24 h incubation. Results are expressed as changes in IL-8 level between VIP-stimulated condition compared with control condition in presence or absence of inhibitors. Means ± SEM of three independent experiments performed in triplicate. $*p < 0.05$, compared with VIP.

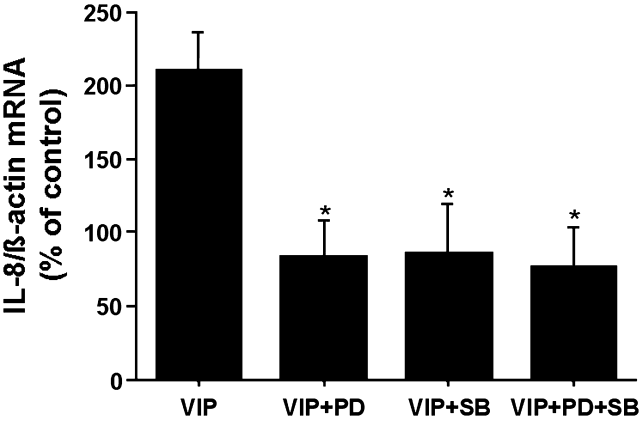

Fig. 5. The VIP-stimulated IL-8 mRNA level involves ERK1/2 and p38 pathways. Analysis of IL-8 mRNA level normalized to β-actin showed that the ERK1/2 inhibitor PD98059 (50 μM) and the p38 inhibitor SB203580 (10 μM) inhibited the IL-8 mRNA level increased by 10^{-9} M VIP after 8 h incubation. Results are normalized to the control level. Means ± SEM of five independent experiments. $*p < 0.03$, compared with VIP.

SB203580 significantly inhibited the VIP-increased basolateral IL-8 secretion by 50% and 25%, respectively. Simultaneous treatment of HT29-Cl.16E cells with both inhibitors reduced the VIP-induced IL-8 secretion by 75%. PD98059 and SB203580 also reduced IL-8 secretion under basal condition (data not shown). Finally, the MAPK inhibitors significantly reduced the VIP-induced increase in IL-8 mRNA level (Fig. 5).

Discussion

This study shows that VIP is able to increase IL-8 secretion and mRNA level in a human colonic epithelial cell line, validated for the study of the VIPergic control of intestinal epithelial functions [2,5,7,8]. This effect of VIP is independent of PKA but involves the MAP kinases ERK1/2 and p38. This study suggests that VIP, a neuromediator present in the majority of enteric neurons projecting to the human mucosa [7], can regulate IL-8 production in vivo.

The increase in IL-8 secretion elicited by VIP is threefold basal secretion in HT29-Cl.16E cells. Similar results were obtained with substance P which increased IL-8 production by about fivefold after 16 h incubation [9]. It can be noted that in our study the magnitude and kinetics of VIP-stimulated IL-8 secretion are different from those observed upon treatment with inflammatory mediators such as TNFα (26-fold basal secretion in the T84 cell line) [14].

We show here that the MAPK ERK1/2 and p38 are involved in the VIP-induced IL-8 secretion and mRNA levels in colonic epithelial cells. These results are consistent with recent studies, which showed that these proteins are involved in cytokine (TNFα and IL-1β)-increased IL-8 production in colonic epithelial cell lines (HT29, Caco-2, and T84) [14,15]. Our study showed that MAPK inhibitors also decrease basal IL-8 secretion, a finding consistent with the decrease in basal IL-8 promoter activity observed after p38 inhibition in Caco-2 cells [15]. The inhibition of both ERK1/2 and p38 did not fully abolish the VIP effect on IL-8 production, suggesting that other intracellular pathways can be involved. Furthermore, we can hypothesize that the cAMP and MAPK pathways could be linked in the VIP-induced IL-8 secretion. Such a link was revealed during activation of H^+, K^+-ATPase by calcitonin in rat kidney cells. Indeed, calcitonin increases intracellular cAMP level which ultimately activates ERK1/2 in rat kidney epithelial cells [19]. In addition, our study shows that VIP increases IL-8 mRNA level in intestinal epithelial cells. Cyclic AMP and MAPK have been reported to modulate IL-8 production through a post-transcriptional mechanism in intestinal epithelial cells [14,16]. Further studies are needed to assess whether the VIP-induced increase of IL-8 secretion occurs at the transcriptional or post-transcriptional level.

Finally we show that VIP modulates HT29-Cl.16E IL-8 secretion in a PKA-independent manner. This finding is consistent with the previous observation that forskolin which, similar to VIP, increases the intracellular cAMP levels, inducing IL-8 secretion in parental HT29 cells via a PKA-independent pathway [20].

The effect of VIP on IL-8 production appears to be different according to the cell type and the environmental conditions, "basal" or inflammatory. Indeed, while VIP stimulates IL-8 secretion in intestinal (our study) and bronchial [21] epithelial cells under basal conditions, VIP reduces IL-8 production in monocytes activated by an inflammatory stimulus, LPS or TNFα [22,23].

In conclusion, this study extends the role of VIP as a key regulator of mucosal functions to the regulation of IL-8 production in human intestinal epithelial cells. The VIP-induced IL-8 secretion could be involved in repair processes of the intestinal epithelium since IL-8 has been shown to stimulate epithelial cell migration and cell growth [12,13,24]. This study further reinforces the role of VIPergic pathways in the maintenance of the human intestinal epithelial barrier.

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